World Journal of Gastroenterology, Hepatology and Endoscopy

Research Article Open Access

Expression of Caveolin-1 Is Linked to Increased Malignancy and Metastasis of Gallbladder Cancer Cells

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Received: 09 Dec 2025
Accepted: 12 Dec 2025
Published: 09 Jan 2026
J Short Name: WJGE

Author Contributions: Oróstica L, Quest AFG, and Lobos-González LL conceptualized and designed the study; Sepúlveda F, Romero D, García P, Díaz J, Rojas-Celis V, Rosa L, Valenzuela S performed experiments; Varas-Godoy M, Gaete-Ramiírez B performed software analysis; Lobos-González LL, Oróstica L validated the results; Varas-Godoy M, Romero D carried out analysis; Sepúlveda F, , Romero D, García P, Diaz J, Rojas-Celis V, Rosa L, Valenzuela S, Oróstica L, Lobos-González LL, and Quest AFG performed data analysis; Roa JC, Valenzuela S contributed resources; Roa JC curated the data; Oróstica L, Lobos-González LL, and Quest AFG prepared the original draft; Lobos-González LL, Montenegro MJ and Quest AFG reviewed and edited the manuscript; Oróstica L carried out visualization; Lobos-González LL and Quest AFG supervised the project and administered research activities; Oróstica L, Lobos-González LL, and Quest AFG acquired funding. All authors approved the final version of the article.

Supported:

FONDAP Project 15130011 and FONDAP continuation FONDAP Continuation #1523A0008; FONDECYT projects 1210644 and 1251598 (AFGQ), and 1211223(LLG); PAI 77190041 and PhD student fellowships (LO).

Keywords:

Gallbladder Cancer; Caveolin-1; E-cadherin; Prognostic Biomarkers; Metastasis; Prognosis; Invasion and Migration; Therapeutic Targets

Citation:

Lorena L Lobos-Gonzalez, Expression of Caveolin-1 Is Linked to Increased Malignancy and Metastasis of Gallbladder Cancer Cells.World Jour of Gastro Hepa Endo. 2026; 31: 1-16

1. Abstract

1.1. Background

Gallbladder cancer (GBC), the most common biliary tract malignancy, shows high incidence in Amerindian Chilean women and poor survival rates. Caveolin-1 (CAV1) may act as a tumour suppressor or promoter depending on E-cadherin (E-cadh) expression, but its specific role in GBC progression remains unclear.

1.2. Aim

To investigate how CAV1 and E-cadh influence metastasis and prognosis in GBC.

1.3. Methods

CAV1 and E-cadh expression were analysed in tumour samples and correlated with clinicopathological features and patient survival. Functional assays included CAV1 overexpression and silencing, as well as E-cadh knockdown experiments in gallbladder cells lines, like GD-b1, to assess effects on migration and invasion in vitro, sub-cutaneous tumour formation and metastasis in vivo in preclinical mouse models.

1.4. Results

CAV1 and E-cadh expression were analysed in tumour samples, and CAV1 was exclusively expressed in tumours and correlated with poor prognosis. High CAV1 together with low E-cadh predicted reduced survival. In vitro assays showed that CAV1 promoted migration/invasion of GBC cells. In vivo xerograph models revealed that CAV1 overexpression in GBC cells increased metastasis and ascites formation, while CAV1 knockdown suppressed tumour spread.

1.5. Conclusion

CAV1 expression favors metastasis of GBCs, especially upon E-cadherin loss and thus represents a candidate therapeutic target in GBC treatments.

1.6. Core Tip

This study identifies CAV1 as a pro-metastatic driver in gallbladder cancer, particularly when E-cadh expression is lost. Integrating patient data, in vitro, and in vivo models, we show that high CAV1 levels are associated with enhanced migration, invasion, and metastasis, and with reduced E-cadh prognosis worsens. These findings highlight CAV1 as a promising therapeutic target for the treatment of aggressive gallbladder cancer.

2. Introduction

Gallbladder Cancer (GBC) is the most common biliary tract cancer. Although, the overall incidence of GBC is not as high as that of other types of cancer, the incidence of this cancer is very high in Indian American populations [1]. For Chile, some of the highest incidence rates in the world are observed. This is particularly true for the female Amerindian (Mapuche) Chilean subpopulation [2]. Prognosis remains poor, as only 10.3% of Chileans diagnosed with GBC survive for more than 5 years, and when this cancer is detected at stage IV, the median survival is only 3.4 months [3]. This dismal outlook is partly explained by late diagnosis, as most gallstone cases-the strongest risk factor-are asymptomatic, leaving patients without warning signs until advanced disease stages [4]. These factors contribute to the status of GBC as a major public health concern in Chile. Typically, this type of cancer is characterized by augmented chronic inflammation due to the release of pro-inflammatory and tumour-promoting cytokines, such as TNF-α and IL-1β, which contribute to tumour progression [5,6]. The presence of such molecules has been linked to GBC progression, as well as the increased expression of proteins, like Caveolin-1 (CAV1), which can reportedly favor tumour progression and metastasis [7-9].

CAV1 has distinct functions in normal and malignant cells. At early stages of tumour development, the protein reportedly inhibits a variety of signalling pathways [10,11], thereby preventing cell transformation and inhibiting tumour growth [12]. Results from our group have linked this ability of CAV1 to the presence of E-cadherin (E-cadh) and the formation of a multiprotein complex that sequesters β-catenin and precludes β-catenin/Tcf-Lef-dependent transcription of genes that favour the development of cancer, such as surviving and cyclooxygenase-2 [13-18].

However, CAV1 also promotes features associated with malignant behaviour, particularly in the absence of E-cadh. After initial silencing of CAV1 during the transformation process, which is often accompanied by the loss of E-cadh, the expression of CAV1 may increase again and is then linked to increased cell migration, filopodia formation, and metastasis [7,9,14,19]. In addition, when CAV1 expression is induced in colon and breast cancer cells lacking E-cadh by treatment with non-cytotoxic doses of antineoplastic drugs that reduce promoter region methylation, CAV1 triggers the development of more malignant and aggressive cancer cell behavior [20]. Acquisition of these traits is linked to enhanced CAV1 phosphorylation of tyrosine-14 [6,18,21–23], as well as downstream activation of a Rab5-Rac1 signaling axis [20,24,25]. Thus, CAV1 plays a dual role in cancer, depending on the presence or absence of E-cadh [18,19].

Available evidence indicates that CAV1 is detected in gallbladder cancer cells but not in healthy gallbladders or gallstones [26]. The statement posits a link between increased CAV1 expression, pro-inflammatory cytokines, and gallstone development, but the relationship is complex and context dependent. While CAV1 has been shown to promote inflammatory signaling in some contexts, it can also inhibit inflammation in others, and its role in cancer development can be both pro- and anti-tumorigenic depending on the cellular and disease-specific environment. For this reason, our general objective was to validate the pro-metastatic effect of CAV1 in GBC, to link this ability to the presence or absence of E-cadh, and to elucidate the signaling pathway (s) associated with these effects.

Here, we provide evidence for enhanced CAV1 expression in gallbladder tissue samples from patients with advanced GBC compared to patients with chronic cholecystitis or a control group with no apparent pathological symptoms. CAV1 presence was clearly associated with a more aggressive GBC cell phenotype in tissue samples. We also observed that CAV1 expression in cell lines increased migration and invasion in in vitro models in a manner that was reduced by E-cadh expression and was dependent on the activation of the Rab5/Rac1 cascade. Finally, we provide evidence that enhanced CAV1 expression in GBC cells promotes metastasis in an in-vivo model. These findings suggest that targeting CAV1-related pathways may improve prognosis and provide new therapeutic opportunities for patients with GBC.

3. Materials and Methods

3.1. Materials

Rabbit polyclonal anti-CAV1 (ab18199 rabbit polyclonal), mouse monoclonal anti-CAV1 (C13620), mouse monoclonal anti-pY14-CAV1 (C611339) and mouse monoclonal anti-E-cad (C61018) antibodies were obtained from BD Transduction Laboratories, NJ, USA; monoclonal anti-β-actin antibodies (sc47778) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA), and all were used as indicated by the manufacturers. Goat anti-rabbit and goat anti-mouse IgG antibodies coupled to horseradish peroxidase (HRP) were obtained from Bio Rad (Hercules Laboratories, San Diego CA, USA). The ECL chemiluminescence substrate and BCA protein determination kit was obtained from Pierce (Rockford, IL, USA). The Plasmid Midi Kit was from Qiagen (Valencia, CA, USA), and human fibronectin was from Becton Dickinson (San Jose, CA, USA). Hygromycin was obtained from Calbiochem (La Jolla, CA, USA), Isopropyl β-D-1-thiogalactopyranoside (IPTG) was from Sigma (St. Louis, MO, USA), and fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA). Cell culture media and antibiotics were from GIBCO (Invitrogen, Carlsbad, CA, USA) and Fugene 6® from Roche Applied Science (Roche, Basel, Switzerland). Mouse monoclonal antibodies anti-caveolin-1 (BD Transduction Laboratories cat. 610407) and antibodies anti-E-cadherin (BD Transduction Laboratories cat. 610182), and mouse monoclonal anti-β-actin (Sigma-Aldrich A5441), which was used as internal control, were all used following the manufacturer's instructions. HRP-labeled secondary antibody to Rabbit or Mouse IgG (KPL, MD, USA).

Plasmids encoding the envelope protein VSV-g (pHCMV-G), the packaging plasmid pD8.9 (pCMVDR8.9) and pLKO.1 plasmids containing shRNA for caveolin-1 and control plasmid containing shRNA for Luciferase (shLuc) were previously described [1].

3.2. Cell Culture

G-415 and GB-d1 gallbladder cancer (GBC) cell lines (wild type (WT), transfected or transduced cells were maintained in RPMI 1640 (Irvine Scientific, Cat. 9161, Santa Ana CA, USA) medium supplemented. PUC-2 GBC cell line was maintained in DMEM-High Glucose Cells. All cell lines used in this study were cultured using a medium containing 10% Fetal Bovine Serum (FBS) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) at 37°C and 5% CO2. EA. hy926 endothelial cells (ATCC, #CRL2922) were maintained in IMEM medium supplemented with 10% FBS and antibiotics in the same concentration mentioned above. GB-d1 and G-415 cell lines were stably transfected with the pLacIOP (referred to as mock) or pLacIOP-Caveolin-1 plasmids (referred to as CAV1, containing the full-length Caveolin-1 sequence, NCBI Reference Sequence: NP_001003296.1) as previously described [2]. Stable cell lines were selected and maintained in culture medium containing 750 μg/mL of Hygromycin. For the expression of E-cad, cells were transiently transfected with the pBATEM2 plasmid lacking a selection marker and encoding murine E-cad under the control of an actin promoter (provided by Amparo Cano, Universidad Autónoma de Madrid, Madrid, Spain) as previously described (29).

3.3. Western Blotting

Protein extraction was obtained from GBC human cell lines cultures using 100 μL RIPA buffer per well (50 mM Tris-Base, 150 mM NaCl, 0.5% Sodium Deoxycholate, 1% Triton X100 and 0.1% Sodium Dodecyl Sulfate). In addition, 1X protease and phosphatase inhibitor cocktail were added. Samples were sonicated and centrifuged at 10,000 g for 20 min at 4°C. The resulting supernatant was used to determine protein concentration with the BCA Protein Assay kit (Thermo Fisher Scientific, Rochester, NY, USA). Total proteins (50 μg) were denatured and fractionated using 12% SDS-PAGE gels. The proteins were transferred to a nitrocellulose membrane and protein transference was confirmed by Ponceau Red S staining. The membranes were blocked for 1 h in Tris-buffered saline with 0.1% Tween-20 (TTBS; 20 mM Tris, pH 7.5, 137 mM NaCl 0.1% Tween 20) containing 5% nonfat dry milk. The membranes were incubated overnight at 4°C using anti-caveolin-1 (dilution 1/3000) or anti-E-cadherin (dilution 1/3000 in block buffer) antibodies. The anti-β-actin was used as internal control (dilution 1/5000 in blocking buffer). The blots were washed with TTBS three times and incubated for 1 h at room temperature with an HRP-labeled second antibody against Rabbit or Mouse IgG (dilution 1/5000 in block buffer). The membranes were washed 3 times for 5 min each with TTBS; the bound antibodies were detected with an enhanced chemiluminescence substrate, Western Lightning Plus-ECL (Perkin-Elmer, Waltham, Massachusetts, USA). Band intensities were quantified by scanning densitometry utilizing an automated digitizing system, UN-SCAN-IT software (version 5.1). The data is presented in Arbitrary Units (AU). The results are expressed as the ratios with respect to the housekeeping protein β-actin.

3.4. Migration and Invasion Assays

Briefly, the inserts were coated on the lower surface with 2 μg/mL of fibronectin as a chemoattractant. Cells (1.5 × 105) re-suspended in serum-free medium were added to the upper chambers, and serum-free medium was added to the bottom chambers. After 16 h, inserts were removed and washed, and cells that migrated to the lower side of the inserts were stained with crystal violet in 2% ethanol and counted in an inverted microscope. Cell migration was evaluated in Boyden Chamber assays (Transwell Costar, 6.5 mm diameter, 8 μm pore size), and invasion was evaluated in Matrigel assays (BD Biosciences, 354,480), as previously reported [27]. These GBC cell lines were used to evaluate migration (for 2 and 5 h) in in vitro assays using Boyden chambers and invasion (for 24 h) in in-vitro assays using inserts with matrigel. Microphotographs show the representative result observed in assays of migration (2 h) and invasion (24 h).

3.5. Trans Endothelial Migration Assay (TEM)

EA. hy926 cells (2.5 x 105) were grown to confluency (after 72 h) on top of an 8-μm-pore size membrane (Transwell; BD Biosciences)[28] for TEM and over coverslips for adhesion assays. B16F10 cells (5 x 104) were labeled with Cell Tracker Green (5 μM; Life Technologies) and added to the top of the trans well inserts for

TEM or added onto endothelial EA.hy926 cell monolayers for adhesion assays. After 24 h, trans well were washed with PBS and wiped with cotton swabs. Inserts and coverslips were fixed with 4% paraformaldehyde in PBS, stained with DAPI and mounted onto glass slides. Migrated green labeled-B16F10 cells were imaged by epifluorescence microscopy (IX81, Olympus). EA. hy926 cells (2.5 x 105) were seeded onto the Trans well inserts and impermeable cell monolayers were allowed to form for 72 h. PUC-2, G-415, and GB-d1 cells (5 x 104), previously stained with Cell Tracker Green (5 μM), were added to the EA.hy926 monolayer in the inserts. Then, GBC cells were allowed to penetrate the EA. hy926 monolayer for 24 h. GBC cells observed by epifluorescence microscopy with a 40X objective on the lower side of the Trans well membrane are shown. Graphs show cells number per optical field for each cell line (mean ± standard deviation). Each bar corresponds to three independent experiments (n = 3). *p <0.05, Kruskal-Wallis Test.

3.6. Animal Studies

Animal studies were conducted in accordance with the appropriate guidelines of the Ethical Committee of the Universidad Del Desarrollo (code UDD-LLG6 and LLG7) [13,15]. Immune-compromised NOD/SCID mice were obtained from Jackson Laboratories (Bar Harbor, Meine, USA) and maintained in the animal facility of the UDD under specific filter air pathogen-free conditions, in a temperature-controlled environment with a 12/12 h light/dark schedule and sterile food and water ad libitum. In the results section, an IC of 95%, a value of significance of 5% or less ($p < 0.05$), and a power calculation of 80% were accepted as statistically significant. All statistical tests were performed using GraphPad Prism 6 software (GraphPad Prism, USA). An r package known as pwr2 was used to calculate the minimum sample size. The "ss.1way" test was used for a one-way balanced ANOVA model (k: number of groups; alpha: level of significance; probability of type I error, beta: probability of type II error (power = 1 − beta); f: effect size; delta: indication of the smallest difference between groups; k, sigma: indication of the standard deviation; and B: number of iterations, where the default value is 100).

3.7. Tumour Growth Assays

Mice BalbC-NoDSciD between 6 and 8 weeks of age were used for experiments (approved by the local bioethics of UDD-LLG6). GBC cell lines: G-415 WT and GB-d1 shCAV1(-), GB-d1 CAV1(+), GB-d1 shE-cadh (-), G-415 E-cadh (+) (5 x 106 cells) in 100 ml physiological saline (0.9% NaCl) were injected subcutaneously into the flanks of mice. Appearance of tumours was monitored by palpitation and was confirmed in initial experiments by histopathological analysis. Tumour growth was monitored via palpitation (volume = width2 × length × π/6), as described [16,20,27]

3.8. Metastasis Assay

BalbC-NoDSciD mice between 6 and 8 weeks of age were used for experiments (approved by the local bioethics of UDD-LLG7) [13,15] and were injected intravenously with G-415 and GB-d1 shCAV1(-), GB-d1 CAV1(+), GB-d1 shE-cadh (-), G-415 E-cadh (+) cells (5 x 106) in 500 μL physiological saline. Then animals were euthanized post-injection days 28 to 30. Tumours were weighed and fixed in PFA 4%. Metastasis was expressed as metastatic tissue mass/total mass in percent (%) post-fixation.

3.9. Subjects and Tissue Samples

The Institutional Review Board of the School of Medicine of Pontificia Universidad Católica approved this study and issued a waiver authorizing the use of archival material without informed consent for samples >2 years old, with the anonymity of patients being preserved (ID GBD 545217, 422962).

This study incorporated 214 advanced GBC samples (pT2, pT3, and pT4) from patients who underwent surgery at the Hernán Henríquez Aravena Hospital (Temuco, Araucanía Region, Chile), 154 chronic cholecystitis samples from patients who underwent cholecystectomy at the Hospital Clínico de la Pontificia Universidad Católica (Santiago, Metropolitan Region, Chile), and 15 normal gallbladder tissue samples from patients without lithiasis disease who underwent surgery for non-biliary-related conditions at the Hospital Sótero del Río (Santiago, Metropolitan Region, Chile). According to Chilean guidelines for the management of biliary tract cancer (BTC) during the recruitment period, none of the included patients with advanced GBC received adjuvant chemotherapy or radiotherapy after surgical treatment. All pT1 cases were excluded due to the absence of follow-up data and the low number of cases. The clinicopathological features of advanced GBC patients were obtained from medical records and are summarized in Table 1.

3.10. Immunohistochemistry

A rabbit polyclonal antibody against human Caveolin-1 (BD Transduction Laboratories, cat 610060, Pleasanton, CA, USA; 1:100 dilution) was used for immunohistochemical analyses. Tissue Microarray Biopsies (TMA) were deparaffinized with Histo-Clear (National Diagnostics, Atlanta, GA, USA) and rehydrated through an ethanol gradient. Heat-induced antigen retrieval was performed by immersing TMA slides in Tris-EDTA buffered saline solution (10 mM Tris, 1 mM EDTA and 0) for 20 min at 99°C in a temperature-regulated bath. Endogenous peroxidase activity was suppressed with phosphate-buffered saline containing 3% H2O2 for 20 min. To prevent non-specific staining, samples were incubated with 5% bovine serum albumin protein solution for 30 min. Then, they were incubated with the primary antibody for 45 min at room temperature and detected with the VECTASTAIN Elite ABC Reagent and Vector Nova RED (Vector Laboratories, Burlingame, CA, USA), according to the manufacturer's instructions. Subsequently, sections were rinsed and counterstained with haematoxylin, dehydrated by intermittent air exposure and Histo-Clear rinses, and finally immobilized on coverslips

3.11. Immunohistochemical Evaluation

A single pathologist (JC Roa) with extensive experience in gallbladder pathology assessed all immunohistochemical staining without knowing the patient's medical records. Levels of CAV1 (15,19, 5) and E-cadh (15,17,5) were evaluated in normal gallbladder, chronic cholecystitis and gallbladder cancer (GBC) tissues.

The total score was the sum of intensity and percentages scores of positive cells. E-cadh expression levels were categorized as Low (0-5) and High (6-7) and CAV1 expression levels as Low (0) and High (>0). Categorization for the expression of each protein was performed based on values below and above the IHC median for each protein.

Samples were considered positive if the total score was greater than 2. GBC tissue samples were classified as CAV1-high if they showed unequivocal staining (≥1+) in more than 50% of neoplastic cells. The remaining samples were classified as CAV1-low or E-cadh-low for survival analysis. This scoring system was previously used in the detection of ENT1 protein in gallbladder tissues [30].

3.12. Transfection of GB-d1cells

The plasmids pLacIOP (referred as mock) and pLacIOP-caveolin-1 (referred as CAV1, containing the full-length dog Caveolin-1 sequence, NCBI Reference Sequence: NP_001003296.1) were previously described[31]. GB-d1 cells were grown to 50-60% confluence in 6 multi-well plates and transfected with 4 μg of pLacIOP-Caveolin-1, using the Lipofectamine 2000 reagent (Invitrogen) as indicated by the manufacturers. After transfection, cells were plated in a complete RPMI medium containing hygromycin (750 μg/mL) for 2 weeks, to yield stably transfected GB-d1 CAV1 cells.

3.13. Lentiviral shRNA, Constructs and Stable Knockdown of Caveolin-1

The oligonucleotide containing shRNA for Caveolin-1 (#5, GCTTCCTGATTGAGATTCAGT) or control shRNA for Luciferase (CGCTGAGTACTTCGAAATGTC) were cloned into the lentiviral vector pLKO.1. To control the shRNA, constructions contained a puromycin resistance gene and the U6 promoter. In order to prepare lentiviral particles (packaging), HEK-293T cells were co-transfected with the pLKO.1 vector and plasmids encoding the packaging plasmid psPax2((#12260, Addgene) and the envelope protein VSV-g (pCMV-VSV-G, #8454, Addgene) using the CaCl2 transfection method [32,33]. After 60 hours of transfection, media was centrifugated at 1000 rpm for 5 min, filtered using a 0.45 mm filter to collect the viral supernatant and centrifugated for 1 h 30 min at 100 000g in a P28S swinging bucket rotor (Hitachi-Himac CP100WX, Japan). The supernatant was discarded, and the particles were resuspended in 200 μL of PBS Lentiviral particles were used to transduce GB-d1 cells. Transduced cells were selected after 72 hours, using 4 mg/ml puromycin for seven days and monitoring its expression by Western blotting [33]. The plasmids used in this study were provided by Dr. Andrew Quest (Universidad de Chile, Santiago, Chile) and Manuel Varas-Godoy (Universidad San Sebastian, Santiago, Chile) described previously [33].

4. Statistical Analysis

Clinicopathological variables, including sex, age (<60 and ≥60 years), ethnicity (Chilean/Latino, Mapuche, other), type of lesion (dysplasia, incipient and advanced GBC), tumour infiltration (muscular, subserosal and serosal) and histological differentiation (poor, well, moderate), were compared among high-CAV1 as well as low- CAV1 and E-cadh expressing patients using the Chi-square test. Survival analysis was done using the Kaplan-Meier method and compared using the log-rank test. Patients with less than 30 survival days were excluded from this analysis. The proportional hazard regression analysis for predictors of survival was assessed with the Cox regression model. Survival analysis was performed in R version 4.2.1 [34]. Significant values were defined as $P < 0.05$. We also analysed the expression trend of CAV1 and E-cadh across dysplasia, incipient carcinoma, and advanced GBC samples using the Jonckheere-Terpstra test. Test function in the R package CLIN fun [35]. Data from blots were analysed with the Kruskal–Wallis test followed by multiple post-test comparisons (Dunn's multiple comparison test) using GraphPad Prism 6 software. In animal experiments, results were compared using unpaired t-tests on means of three or more independent values (animals) and corroborated with a Duncan test. Values averaged from at least three independent experiments were compared. The value of $P < 0.05$ was considered significant.

5. Results

5.1. Gallbladder Cancer Is Associated with Increased Caveolin-1 and Decreased E-Cadherin Expression Levels

Immunohistochemical analysis was used to evaluate the expression of CAV1 and E-cadh in tissue samples from patients at different cancer stages and in control samples. E-cadh was readily detectable in normal gallbladder tissue and in chronic cholecystitis, which served as controls. However, E-cadh levels were somewhat reduced in the gallbladder tumour tissue. In contrast, CAV1 was only detected in tumour samples and was absent in control samples (Figure 1A). Importantly, there were notable differences in E-cadh distribution between the control and tumour samples. In the control samples, E-cadh localized exclusively to the cell periphery, whereas in the tumour samples, it exhibited a more cytoplasmic distribution. (Figure 1A, lower panel).

We also analysed the percentage of cells at different stages that exhibited different signal levels (Figure 1B). Approximately 60% of the tumour cells were strongly positive for CAV1, 35% showed moderate levels, and only 5% were negative for CAV1. In the control samples, 100% of the epithelial cells showed a strong signal for E-cadh, whereas levels were reduced in 20% of the tumour cells.

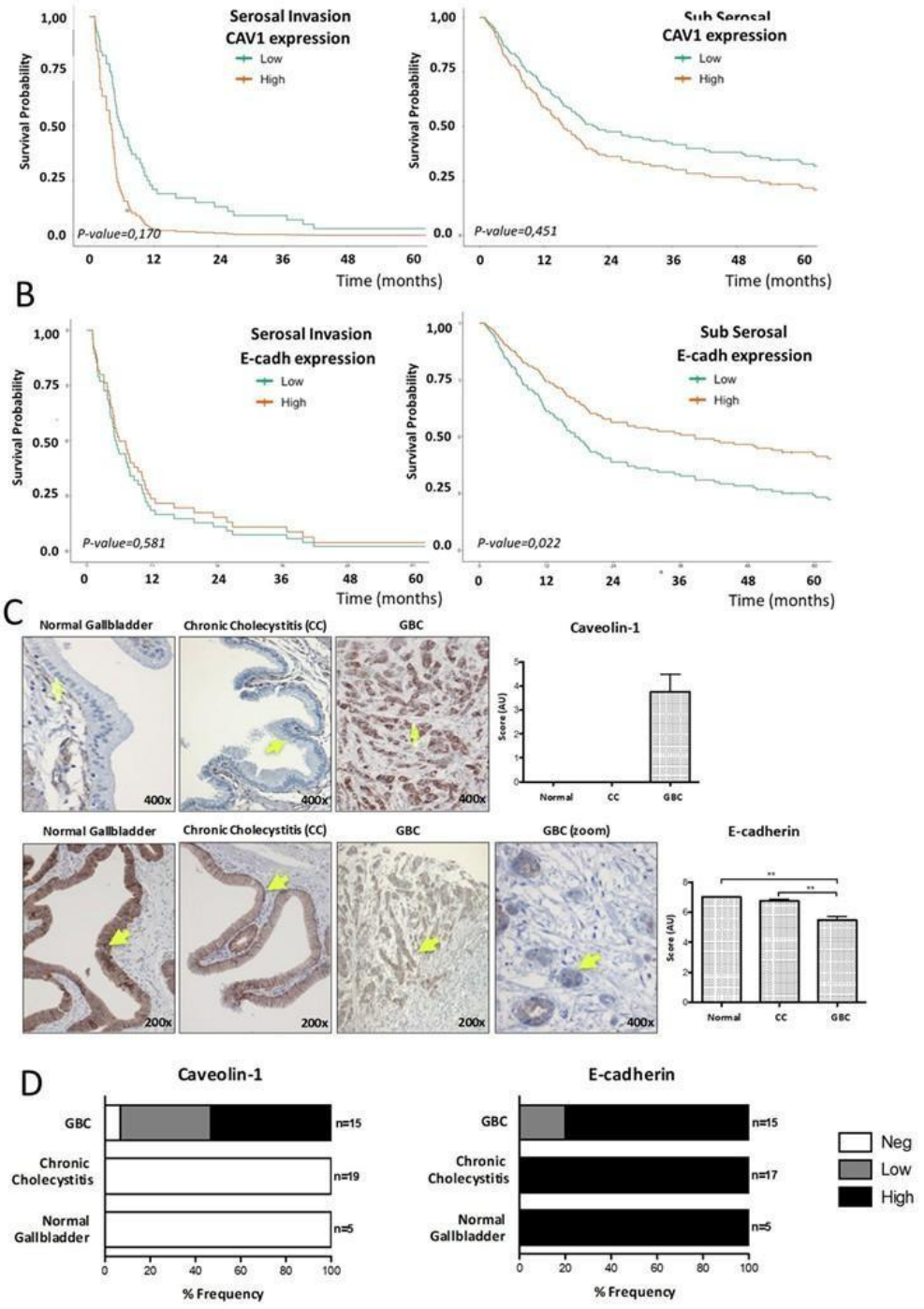

Figure 1; Caveolin-1 and E-cadherin levels in gallbladder tissue samples. A: Overall survival of patients with GBC and high- or low-CAV1 expression and serosal or subserosal infiltration. B: Overall survival of GBC patients expressing high- or low-Ecadh levels with serosal or subserosal infiltration. B: CAV1 and E-cadh protein levels in gallbladder tissue samples from control patients, patients with chronic cholecystitis (CC) and GBC patients were analyzed by immunohistochemistry. Representative micrographs of gallbladder tissue sections from the three study groups are shown: Normal (n = 5), Chronic Cholecystitis (n = 19), and Gallbladder Cancer (GBC) (n = 15). The presence of CAV1 or E-cadh in the epithelial and stromal compartments of gallbladder tissues was visible as brown staining in tissue samples. Top panel: Green arrows indicate the epithelial tissue, which was negative for CAV1 in Normal and Chronic Cholecystitis samples, while in the tumor epithelium (Gallbladder Cancer group), CAV1 was readily detected. Bottom panel: Green arrows indicate markedly positive staining for E-cadh in the epithelial tissue of the normal and Chronic Cholecystitis groups. In GBC, tumor epithelium (green arrow), E-cadh staining was notably reduced compared to that in the other groups. At larger magnification, E-cadh observed in GBC samples was more cytoplasmic than in normal and chronic cholecystitis samples. Graphs showing the semi-quantitative evaluation of the protein levels: Total Score indicating intensity and presence (% of positive cells) in the tissue samples. Data are shown as the mean in arbitrary units (AU) ± SEM. **P < 0.01 analyzed by non-parametric Krukal-Wallis test and Dunn's post-test. C) Frequency of CAV1 and E-cadh presence in gallbladder tissues from Normal, CC and GBC groups. Bar graphs show the % Frequency of the Total Score (in A) Neg (negative): Total Score of 0-2; Low: Total Score of 3-4; High: Total Score of 5-7. An experienced pathologist performed the analysis.

5.2. Caveolin-1 and E-Cadherin Protein Levels at Different Stages of GBC Progression and Their Correlation with Patient Survival

In the 226 patient samples available, we measured CAV1 expression in 209 (92.5%) and E-cadh expression in 210 (92.9%), respectively. We associated these expression levels with the clinicopathological and sociodemographic characteristics of patients. Younger patients (<60 years old) exhibited higher CAV1 expression than older patients. Interestingly, higher E-cadh expression was observed in advanced GBC and incipient carcinoma cases than in dysplasia cases (Table 1). Moreover, a significant increasing trend in E-cadh IHC scores was observed across dysplasia, incipient carcinoma, and in advanced GBC (P-trend = 0.029 the protein was mainly cytoplasmic.

We also analyzed overall survival (OS) among relevant clinicopathological variables in patients with advanced GBC. The median survival was 6.3 months for serosal infiltration and 22.1 months for subserosa infiltration (Figure 1A). On the other hand, elevated E-cadh expression correlated with increased OS, with an HR of 0.69 (95%CI: 0.49-0.96; Figure 1B and Table 2). Patients with low E-cadh expressions had A worse OS (median survival of 17.1 months), whereas those with high expressions had a median survival of 38.6 months (Figure 1B). No significant associations were found either between E-cadh expression and overall survival in serosal infiltration cases, or between CAV1 expression and OS (Figure 1A-B).

Table 1: Clinicopathological characteristics among high- and low- CAV1 and E-cadh tissues.

		†(Caveolin-1 (N=209			†(E-cadherin (N=210		
Clinic pathological Features	**Patients (N=226) (%) n**	**Low (N=179) (%) n**	**High (N=30) (%) n**	***P-value*††**	**Low (N=122) (%) n**	**High (N=88) (%) n**	***P-value*††**
Sex				>0.9			0.08
Women	194 (85.8)	151 (84.4)	26 (86.7)		110 (90.2)	71 (80.7)	
Men	32 (14.2)	28 (15.6)	4 (13.3)		12 (9.8)	17 (19.3)	
Age				**0.023**			>0.9
<60 years	73 (32.3)	54 (30.2)	16 (53.3)		40 (32.8)	30 (34.1)	
≥60 years	153 (67.7)	125 (69.8)	14 (46.7)		82 (67.2)	58 (65.9)	
Ethnicity				0.4			0.9
Chilean/Latino	154 (68.1)	116 (64.8)	23 (76.7)		87 (71.3)	61 (69.3)	
Mapuche	30 (13.3)	26 (14.5)	3 (10.0)		16 (13.1)	11 (12.5)	
Other	42 (18.6)	37 (20.7)	4 (13.3)		19 (15.6)	16 (18.2)	
Lesion				0.1			**0.039**
Dysplasia	14 (6.2)	12 (6.7)	0 (0)		11 (9.0)	1 (1.1)	
Incipient GBC	13 (5.8)	10 (5.6)	0 (0)		8 (6.6)	4 (4.5)	
Advanced GBC	199 (88.1)	157 (87.7)	30 (100)		103 (84.4)	83 (94.3)	
Tumor infiltration*				0.07			0.7
Muscular	13 (6.1)	10 (5.6)	0 (0)		8 (6.6)	4 (4.5)	
Subserosal	133 (62.7)	110 (61.5)	16 (53.3)		69 (56.6)	57 (64.8)	
Serosal	66 (31.1)	47 (26.3)	14 (46.7)		34 (27.9)	26 (29.5)	
Histological differentiation*				0.5			0.7
Poor	56 (26.4)	43 (24.0)	11 (36.7)		28 (23.0)	23 (26.1)	
Well	108 (50.9)	85 (47.5)	14 (46.7)		54 (44.3)	46 (52.3)	
Moderate	35 (16.5)	29 (16.2)	4 (13.3)		21 (17.2)	13 (14.8)	

*incipient and advanced GBC (N=212; 100%).

†17 and 16 patients were not analyzed for CAV and E-cadh expression, respectively, due to inadequate IHC staining.

††chi-square test for categorical comparisons. Significant differences (*P-value<0.05*) are indicated in bold.

Table 2: Hazard ratios (HRs) and 95% confidence intervals (CIs) for overall survival among clinicopathological variables in advanced GBC cases.

Variable	n (%)	Beta coefficient	HR (95% CI)*	*P-value*†
Sex				
Women	155 (86.1)	Reference	-----	-----
Men	25 (13.9)	-0.03	0.97 (0.61-1.56)	0.9
Age				
<60 years	62 (34.4)	Reference	-----	-----
≥60 years	118 (65.6)	0.10	1.11 (0.79-1.56)	0.563
Ethnicity				
Chilean/Latino	122 (67.8)	Reference	-----	-----
Mapuche	26 (14.4)	0.09	1.10 (0.69-1.74)	0.692
Other	32 (17.8)	0.04	1.04 (0.67-1.60)	0.869
Tumor infiltration				
Sub-serosal	125 (69.4)	Reference	-----	-----
Serosal	55 (30.6)	1.23	**3.44 (2.41-4.90)**	**<0.001**
Histological differentiation††				
Poor	47 (26.1)	Reference	-----	-----
Well	89 (49.4)	0.07	1.07 (0.73-1.58)	0.731
Moderate	34 (18.9)	-0.27	0.76 (0.47-1.24)	0.270
Caveolin expression††				
Low	147 (81.7)	Reference	-----	-----
High	23 (12.8)	0.37	1.45 (0.91-2.31)	0.117
E-cadherin expression††				
Low	90 (50.0)	Reference	-----	-----
High	78 (43.3)	-0.38	**0.69 (0.49-0.96)**	**0.030**

5.3. Elevated Levels of CAV1 and Reduced Levels of E-Cadh Are Associated with Enhanced Migration and Invasion of Gallbladder Cancer Cells

To evaluate the relevance of CAV1 and E-cadh in GBC, three gallbladder cancer cell lines (G-415, GB-d1, and PUC-2) with different CAV1/E-cadh expression levels were chosen to determine invasion, migration, and trans-endothelial migration in vitro. First, the expression of both proteins in the three cell lines was evaluated by Western blotting (Figure 2A). CAV-1 levels were substantially higher in G415 cells than in PUC-2 cells and in GB-d1 cells the protein was almost undetectable. Alternatively, for E-cadh, the inverse situation was observed. PUC-2 had the highest E-cadh protein levels, followed by G-415 cells, whereas in GB-d1 cells the protein was almost undetectable.

As anticipated, based on our previously reported findings in other cancer cell lines, G-415 cells, with the highest CAV1 and lowest E-cadh levels, displayed the greatest capacity to migrate and invade in vitro, while for GB-d1 and PUC-2 cells, migration and invasion were significantly reduced (Figure 2B). In trans-endothelial migration assays, a similar behavior was observed (Figure 2C). Taken together, these results indicated that higher levels of CAV1 and reduced levels of E-cadh in GBC are associated with a more aggressive cellular phenotype.

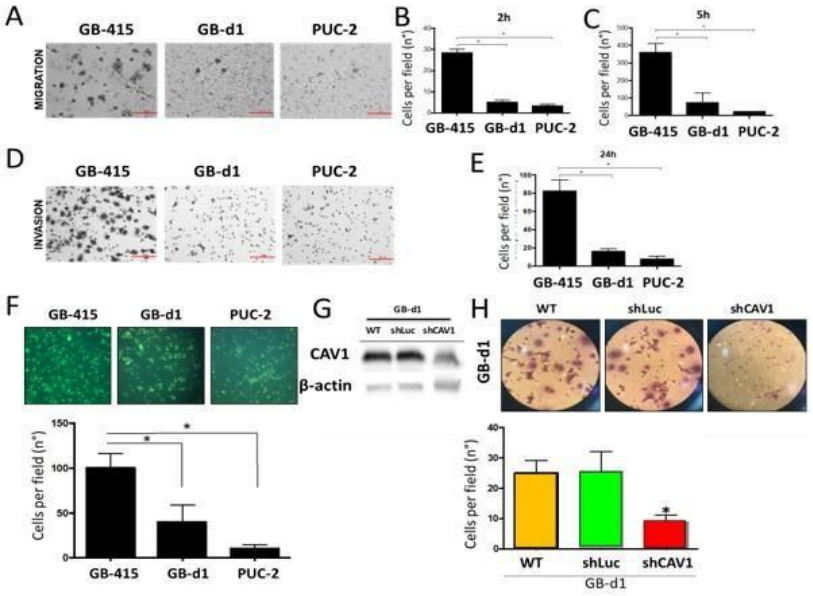

Figure 2: Elevated CAV1 and reduced E-cadh levels enhance migration, invasion, and trans-endothelial migration of GBC cells.
A: Western blot analysis shows differential CAV1 and E-cadh protein levels in G-415, GB-d1, and PUC-2 cells. Quantifications (mean ± SEM, n=3) reveal an inverse relationship between CAV1 and E-cadh levels (*p < 0.01, Kruskal-Wallis). B: Migration and invasion assays demonstrate increased capacities in G-415 cells compared to GB-d1 and PUC-2, consistent with their CAV1/E-cadh expression profiles (*p < 0.05). C: Trans-endothelial migration assay highlights enhanced penetration by G-415 cells, quantified as mean ± SD (n=3, *P < 0.05). D, E: Knockdown of CAV1 in GB-d1 cells (shCAV1) reduces invasion compared to wild-type (WT) and control (shLuc) cells, confirmed via Western blotting and Matrigel assays (*p < 0.05). F) Overexpression of CAV1 in GB-d1 cells (CAV1+) increases migration and invasion compared to control (Mock) cells, as shown by Western blotting and in vitro assays (*p < 0.05). G). Knockdown of E-cadh (shE-cadh) in GB-d1 cells enhances invasion, demonstrating the inhibitory role of E-cadh in GBC cell aggressiveness (*p < 0.05). Data shown are averages from three independent experiments.

5.4. CAV1 Promotes Migration and Invasion of Gallbladder Cancer Cells

To link directly the expression of CAV1 to the ability of GBC cells to migrate and invade, we reduced CAV1 levels in GB-d1 cells using lentiviral particles with a shRNA construct against CAV-1. The protein levels of CAV-1 in GB-d1 cells transduced with a control shRNA (shLuc) were similar to observed by Western blotting for wild-type cells, while in cells transduced with shRNA against CAV1 (shCAV1), CAV1 levels were reduced by approximately 4-fold. Consistent with our previous observations, GB-d1(shCAV1) cells invaded significantly less than wild-type and shLuc cells (Figure 3A).

GB-d1 cells were then transfected with a plasmid that permitted CAV-1 overexpression. As revealed by Western blotting, GB-d1(-CAV1) cells expressed significantly higher levels of the protein than the GB-d1(Mock) cells (Figure 3C). Moreover, elevated CAV1 expression resulted in increased migration compared to that observed for the control cells (Mock) (Figure 3D). These observations provide direct evidence that elevated levels of CAV1 are linked to more aggressive behaviour of GBC cells in in vitro assays.

5.5. Elevated CAV1 Levels and Reduced E-Cadh Expression Were Found to Enhance Migration, Invasion and Trans-Endothelial Migration of GBC Cells

Our analysis revealed differential CAV1 and E-cadh protein expression in the three GBC cell lines, G-415, GB-d1, and PUC-2. Quantification of protein levels, normalized to β-actin, demonstrated significant differences between cell lines, hen data were averaged from three independent experiments.

Functional in vitro assays indicated that GBC cells exhibit increased migration after 2 and 5 h as well as invasion after 24 h. Representative microphotographs confirmed these findings, with quantitative analysis revealing significant differences in the number of migrating and invading cells (Figure 2). Trans-endothelial migration was evaluated using EA. hy926 monolayers, showing that GBC cells penetrated the endothelial barrier more efficiently, as observed by epifluorescence microscopy (40× magnification). Manipulating CAV1 levels in G-415 cells further supported these findings. Cells with reduced CAV1 expression (shCAV1 transduction) demonstrated significantly lower invasive capacity compared to wild-type (WT) or control-transduced cells (shLuc), as shown in in-vitro invasion assays. Conversely, for GB-d1 cells overexpressing CAV1 (pLacIOP-CAV1 transfection) migration and invasion increased compared to mock-transfected cells, as confirmed by Boyden chamber and Matrigel assays, respectively.

Similarly, reducing E-cadh expression in GB-d1 cells (shE-cadh transduction) significantly increased the invasive potential in Matrigel assays compared to WT cells. Quantitative analysis confirmed that reduced E-cadh expression is associated with enhanced invasive behaviour, reinforcing the role of E-cadh downregulation in promoting GBC cell invasiveness.

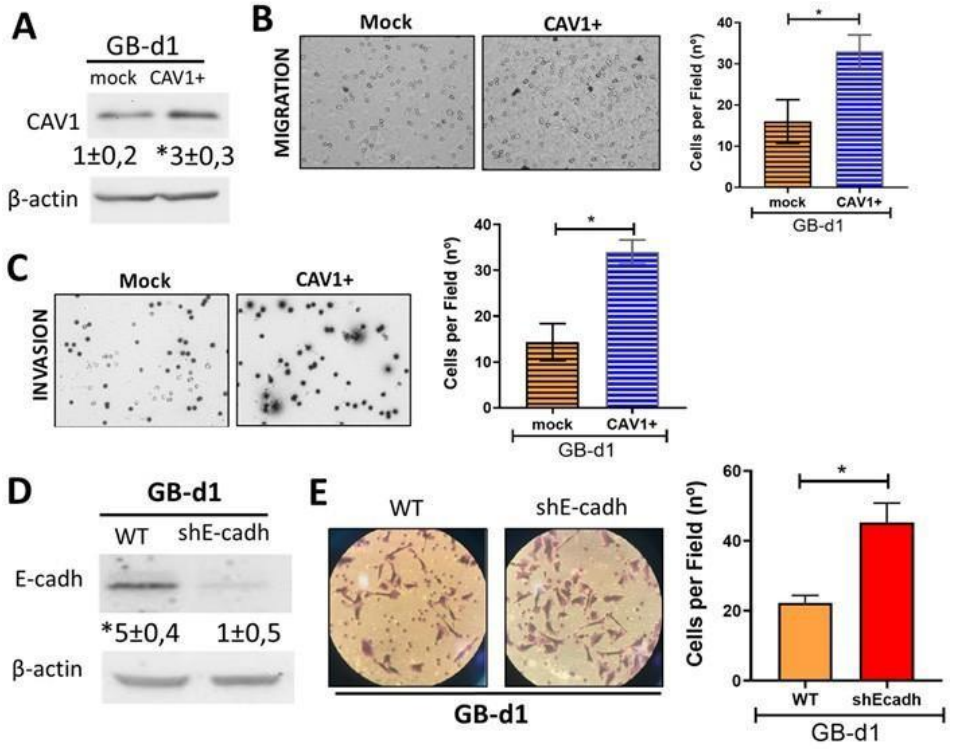

Figure 3: Elevated CAV1 and reduced E-cadherin levels promote tumoroid formation of GDB1 gallbladder cancer cells in 3D culture.
The GB-d1 cells were cultivated in a minimum medium to evaluate the potential presence of cancer stem cells. The cells were maintained for 14 days without change of medium or supplement, such that only the cells with stem cell capacity were able to grow. After this period tumoroids were filtered through a cell strainer and A: the diameter was measured (in µm). Averages indicated are the mean ± SD B: the total number of colonies (over 100 µm) was determined. C: Representative photographs are shown below the graph (Magnification Bar 500 um).

5.6. E-Cadh Reduces Migration and Invasion of Gallbladder Cancer Cells

On the other hand, the relationship between E-cadh levels and in-vitro invasion was evaluated. For this, GB-d1 cells transfected with shRNA against E-cadh were used, and invasion was evaluated. The results revealed that GB-d1 shE-cadh cells had lower E-cadh levels than wild type cells as determined by Western blot analysis. Furthermore, shE-cadh cells were found to invade more than control cells (Figure 2).

CAV1 levels were determined by Western blotting in GB-d1 wild type cells (WT), GB-d1 cells stably transduced with a short hairpin RNA against CAV1 expression (shCAV1) and GB-d1 cells stably transduced with a short hairpin RNA against the luciferase gene (shLuc). Blots revealed lower CAV1 levels in GB-d1 shCAV1 cells than in GB-d1 WT and shLuc control cells. These cells were then used to evaluate 3Dinvasion in matrigel transwell assays. Microphotographs revealed elevated presence of GB-d1 WT and shLuc cells in each insert compared to shCAV1 cells (Figure 2D-upper images). The graph shows the quantification of the cell number per optical field for each cell type. These results indicated that cells with reduced CAV1 levels have a lower invasive capacity in in-vitro assays than cells with a higher CAV1 content.

5.7. CAV1 Expression Promotes Metastasis In-Vivo of Gallbladder Cancer Cells

In the following assays, we evaluated how the presence of CAV1 and E-cadh proteins affects the metastasis of GBC cells using an in vivo animal model. Wild type cells and cells transfected to overexpress, or cells silenced for CAV1 were used in these assays. Furthermore, the role of E-cadh in GBC cell metastasis was evaluated using cells with silenced E-cadh. The results show that G-415 wild type cells with higher CAV1 levels formed tumours to a greater extent in ganglia. Furthermore, for animals inoculated with G-415 cells more malignant ascites and increased abdominal volume were detected, both representing parameters associated with enhanced metastasis (Figure 3). When E-cadh is knock-down (like in Figure 6D), the suppressor tumour effect is lost, and this is a expected results because y E-cadh is a tumour suppressor in presence of CAV1 this role are increasing, but in the absence the roles are lost.

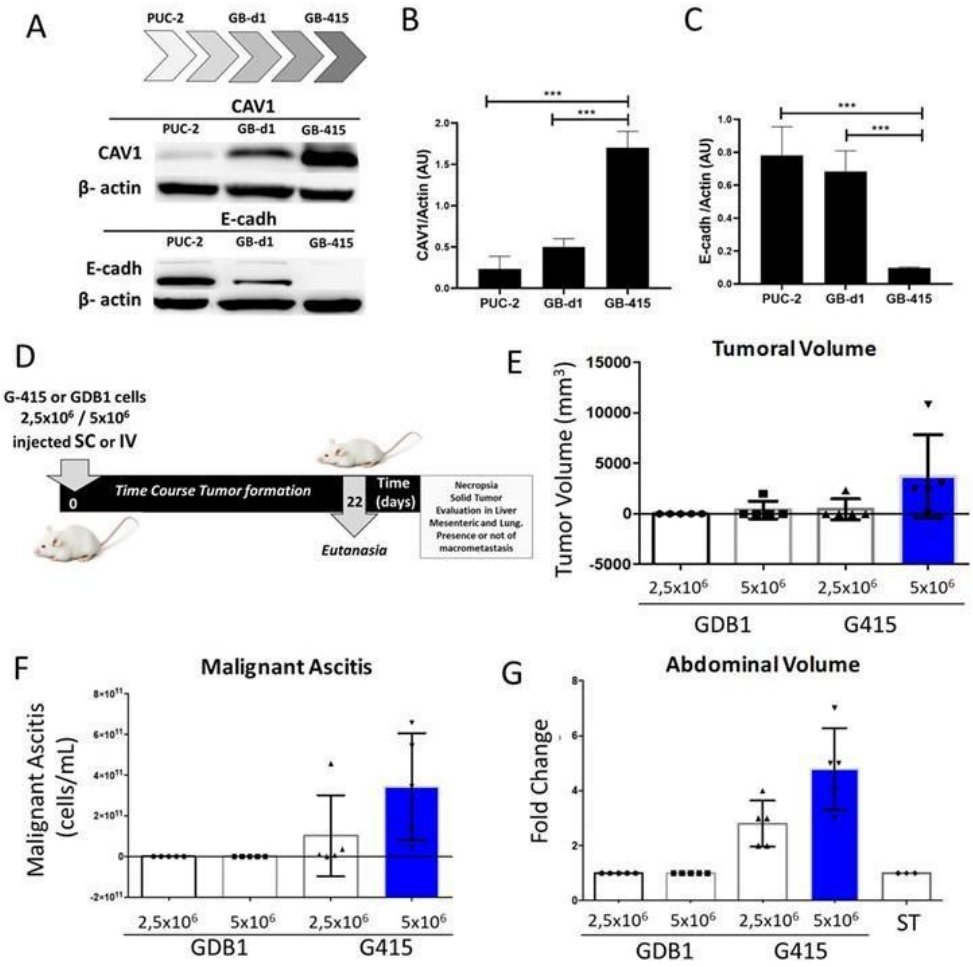

Figure 4: Comparative Wild Type Cell Line behavior: elevated CAV1 and reduced E-cad levels enhance tumor formation and metastasis in gallbladder cancer cells.

GBC wild type cell lines were used to assess tumor formation and/or metastasis in an in-vivo model. A) Western blot analysis shows differential CAV1 and E-cadh protein levels in G-415, GB-d1, and PUC-2 cells. Quantifications of B) CAV1 and C) E-cadh (mean ± SEM, n=3) reveal an inverse relationship between protein levels (***P < 0.01, Kruskal-Wallis). In addition, to determine the effect of elevated CAV1 levels on tumor formation and/or metastasis, GB-d1 and G-415, wild type cells were used for evaluation in an in vivo model. D) Each cell type was inoculated by intraperitoneal injection into the animals. After 30 days, necropsy was performed and tumor formation and/or parameters associated with metastasis were evaluated in immune-suppressed BalbC-NoDSciD mice. Scheme showing the time course of the in vivo assays E) Tumor volumes (mm3) measured (day 30) for five mice per group are shown. Averages shown are the means ± SD *P <0.05, Kruskal-Willis Test. And the abdominal volume of animals with malignant ascites was determined using a curve measuring tape. F) Malignant ascites, determined as the number of cells obtained per 1 mL of abdominal fluid in animals inoculated with GDB1 and G415 cells (mean ± SD, *P <0.05, Kruskal-Wallis Test) were compared with basal data obtained from healthy animals of the same age and weight (ST group). G) fold change of metastasis is indicated (mean ± SD, *P <0.05, Kruskal-Wallis Test).

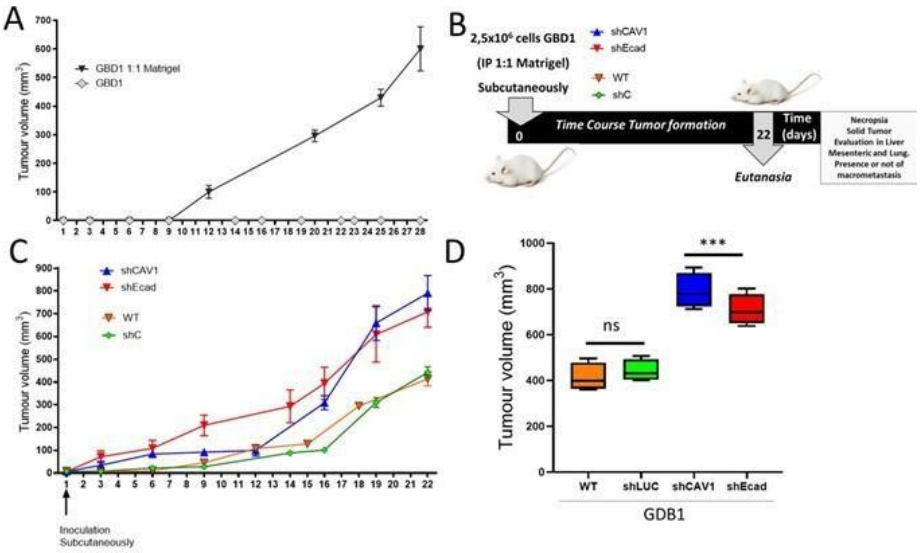

Figure 5: Elevated Caveolin-1 (CAV1) and reduced E-cadh levels enhance migration and invasion of GB-d1 gallbladder cancer cells. A: Western blot analysis showing differential CAV1 protein levels in GB-d1, GB-d1(mock) and GB-d1(CAV1+) sub-line cells. CAV1 Quantifications (mean ± SEM, n=3). B: Migration assays demonstrating increased capacities in GB-d1(CAV1+) compared to GB-d1(mock) (*P < 0.05). C: Overexpression of CAV1 in GB-d1(CAV1+) increases invasion compared to GB-d1(mock) cells (*P < 0.05), showed representative crystal-violet stained and cells per field counted. D: A representative Western blot showing knockdown of E-cadh in GB-d1(shE-cadh). E-cadh levels decreased 5 times compared with GB-d1 wildtype cells (E-cad quantifications, mean ± SEM, n=3). E: Enhanced invasion of GB-d1(shE-cadh) cells in matrigel assays. Image shows representative fields of crystal-violet stained cells (left) and average numbers of cells per field (right) from three independent experiments (mean ± SEM, n=3; *P < 0.05).

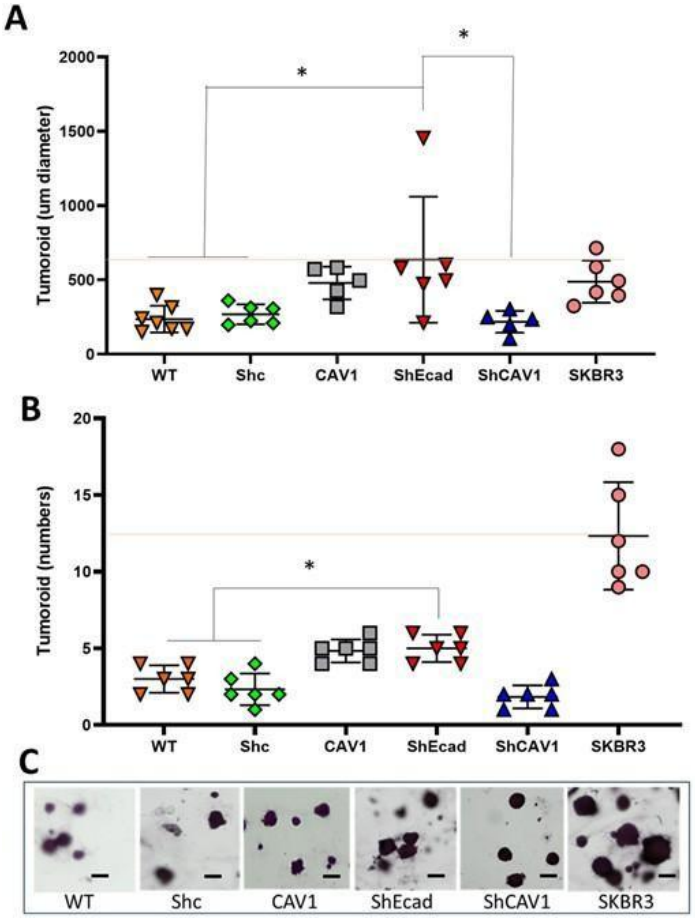

Figure 6: Elevated CAV1 or reduced E-cadh levels promote tumor formation by GDB1 gallbladder cancer cells in a xenograft model.

A: Immune-suppressed BalbC-NoDSciD mice were subcutaneously inoculated with 2.5 × 10 G⁶ DB1 cells; one group of 3 animals received the cells alone, while the other group received the cells mixed in a 1:1 ratio with Matrigel Geltrex. **B:** Schematic representation of the timeline for the subcutaneous tumor formation assay. **C:** Time-course of tumor formation in four groups of animals (5 mice per group), all inoculated with GDB1 cells are shown; wild-type cells (orange), cells transduced with a control shRNA (shControl, green), cells with silenced CAV1 (shCAV1, blue), and cells with silenced E-cadh (shEcad, red). **D:** At the end of the experiment (day 22), tumor masses were determined (mean ± SD, ***P < 0.001).

6. Discussion

This study began with the hypothesis that Caveolin-1 (CAV1) contributes to gallbladder cancer (GBC) progression and metastasis, particularly when E-cadherin (E-cadh) is absent. Our results strongly support this, showing that CAV1 is absent in normal and chronic cholecystitis gallbladder tissues, but consistently present in tumour samples. These observations are indicative of a binary ('all-or-nothing') pattern of expression specific to malignant transformation, which highlights its potential clinical utility as a biomarker to distinguish malignant from benign gallbladder lesions. In contrast, E-cadh showed progressive mis localization and reduction from control to cancerous tissue, in agreement with previous studies linking E-cadh loss to epithelial-mesenchymal transition (EMT) and poor prognosis [17].

Epidemiologically, GBC presents alarming mortality rates, especially in Chilean women of Mapuche ancestry, where 5-year survival is below 10%, and median survival in stage IV is only 3.4 months [3]. Our patient cohort reflects these outcomes, with low survival in advanced-stage cases and a clear correlation between high E-cadh expression and improved overall survival, reinforcing its protective role. These findings suggest that assessing E-cadh expression may have prognostic value for patient survival in GBC.

Regarding in vitro experiments, we selected the GB-d1 cell line for further experimentation due to its nearly undetectable levels of both CAV1 and E-cadherin. This allowed us to manipulate each protein independently and study their individual and combined contributions to migration, invasion, and metastasis.

In vitro, elevated CAV1 and reduced E-cadh levels were consistently associated with more aggressive behaviours, including enhanced migration, invasion, and trans-endothelial migration. These phenotypes were validated through shRNA-mediated knockdown and overexpression assays. Our results are in agreement with observations in melanoma and breast cancer models, where phosphorylated CAV1 (Y14) activates Rac1 via Rab5, promoting motility [6,18,19,22,36,37]. Such mechanisms may explain the highly invasive clinical behaviour of GBC, and point to the CAV1/E-cadh status as a potential predictor of GBC aggressiveness

While valuable, the in vitro models used lack immune and stromal components and do not recapitulate the inflammatory context of human GBC. However, based on our results, we hypothesize that in cells co-expressing CAV1 and E-cadh, the two form a complex that sequesters β-catenin at the membrane [17], suppressing oncogenic transcription events (CAV1 as a tumor suppressor). When E-cadh is lost, CAV1 becomes phosphorylated on Y14 [6,22,23] and drives pro-metastatic signaling. This shift in the CAV1 function likely explains the context-dependent duality observed in vitro [7] and in vivo [16,21,38]

Our in vivo experiments agree with these observations. Mice injected with CAV1-overexpressing cells showed increased tumour burden, malignant ascites, and peritoneal metastasis, while CAV1 silencing suppressed such behaviour. When in the bladder cancer cells decreased the CAV1 protein levels, increasing the tumour formation, these results are agreed with the fact that CAV1 can also act as a tumour suppressor in the absence of E-cadh by preventing HIF1a induction and suppressing UPR. Findings consistent with the reported increase in lung (and liver) metastases in melanoma, including a tumour resection model that imitates clinical practice[18]. These results underscore CAV1's role as a driver of metastatic progression in GBC. Notably, E-cadh knockdown alone was sufficient to increase metastasis, reinforcing the idea that CAV1 function is context-dependent and modulated by E-cadh availability [29,39]. Clinically, this duality suggests that evaluating both markers together could help identify patients at risk for early dissemination.

Thus, CAV1 overexpression enhanced metastatic dissemination, ascites formation, and tumour burden, whereas silencing CAV1 or restoring E-cadh reduced these outcomes [29,40]. This pattern is consistent with the clinical presentation of GBC, which is known for its high rate of peritoneal dissemination and aggressive behaviour.

A crucial question arising from our study is what induces CAV1 upregulation in GBC cells. Chronic inflammation is a hallmark of GBC. In this context, literature suggests a link to pro-inflammatory cytokines such as TNF-α and IL-1β, which are abundant in GBC and promote CAV1 expression [9,10]. Exploring this link may reveal novel therapeutic targets in inflammation-driven GBC progression. A key limitation of our study is the use of immune-compromised mouse models [41], which do not fully recapitulate the inflammatory tumour microenvironment observed in human GBC. Future clinical studies should examine whether CAV1 expression correlates with systemic inflammatory markers in patients, potentially enabling its use in routine diagnostic or prognostic assessments.

Altogether, these findings underscore the clinical relevance of CAV1 and E-cadherin as potential biomarkers for patient stratification in GBC. Detecting CAV1 overexpression in the absence of E-cadh may help identify patients at higher risk of early dissemination and poorer survival [19]. Moreover, targeting CAV1-related pathways could provide a novel therapeutic approach in a setting where effective treatments are lacking [17]. Future studies should validate these results in larger patient cohorts and assess their utility in clinical practice.

In summary, our findings support a model (Figure 7) in which a pro-inflammatory environment downregulates E-cadh and induces CAV1.This work provides compelling evidence that CAV1 is not only a marker of poor prognosis in GBC, but also a mechanistic driver of metastasis, particularly when E-cadh is lost [42]. These findings support the use of the CAV1/E-cadh status as a potential prognostic biomarker and both proteins as therapeutic targets, opening opportunities for more personalized treatment strategies in GBC patients.

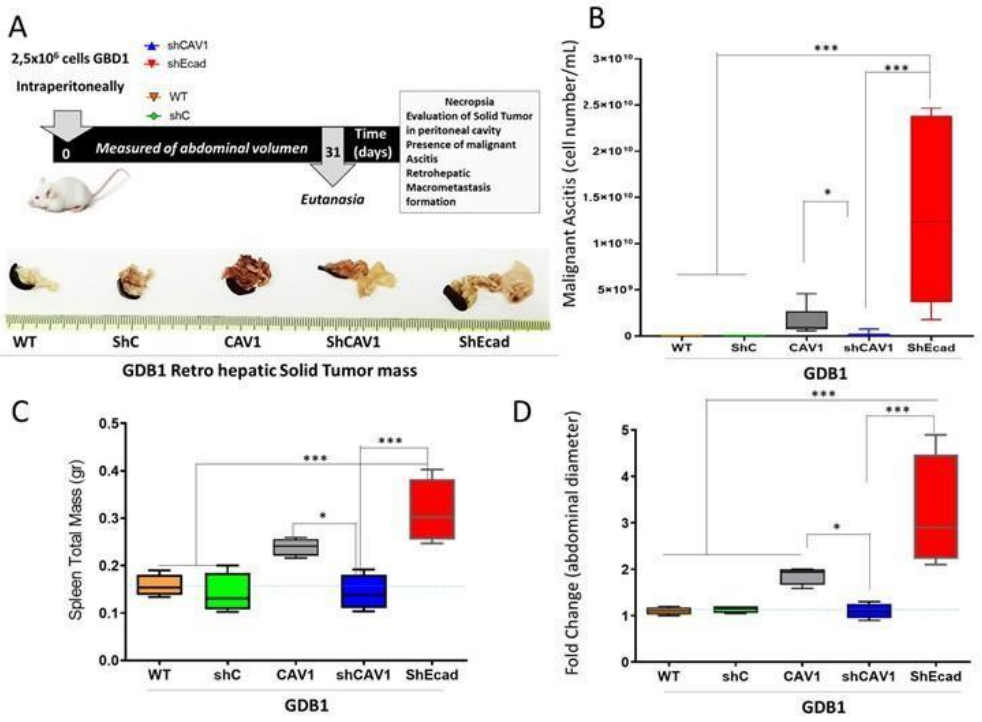

Figure 7: Elevated CAV1 and reduced E-cadh levels induce metastasis of GDB1 gallbladder cancer cells in a xenograft model. A: Schematic representation of the timeline for the intraperitoneally carcinomatosis tumor formation assay. After 21 days, the BalbC-NoDSciD mice were euthanized and the solid tumor mass adherent to the spleen was measured: GDB1 retro hepatic solid tumor mass. B: in all mice the presence or absence of ascites was evaluated and for GDB1(cav1), GDB1(shCAV1) and GDB1(shE-cadh) tumors the cell number per mL for each mouse was determined (mean ± SD; *P < 0.05, ***P < 0.001). C: the total spleen mass is shown, the changes observed were due to the solid tumor mass adherent to the spleen (mean ± SD; *P < 0.05, ***P < 0.001). D: The accumulation of malignant ascites as reflected in changes in the abdominal diameter were evaluated for the different groups of mice (mean ± SD; *P < 0.05, ***P < 0.001). Orange represents wild-type cells, green corresponds to cells transduced with a control shRNA (shControl), blue indicates cells with silenced CAV1 (shCAV1), and red denotes cells with silenced E-cadh (shEcadh).

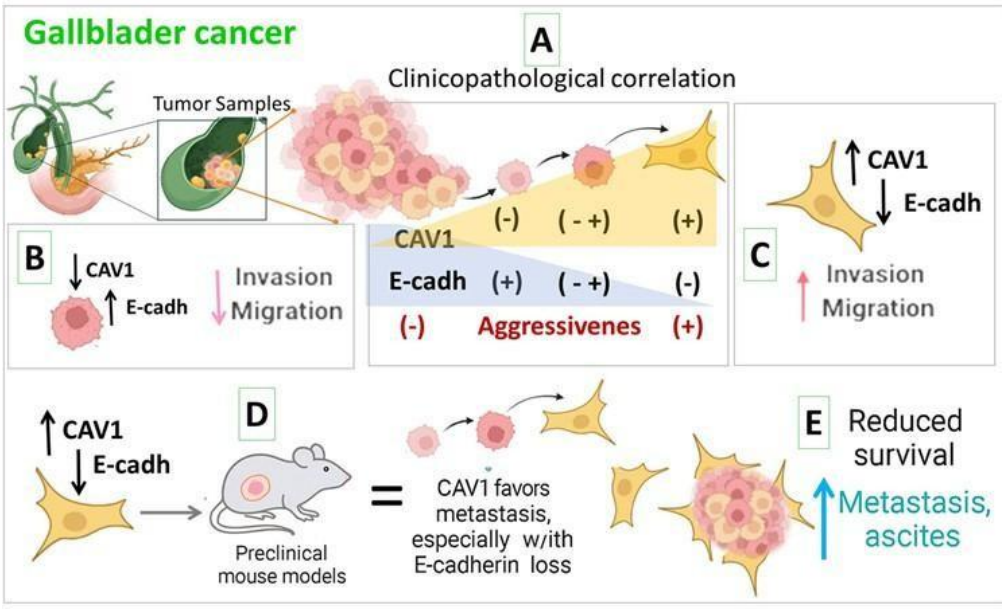

Figure 8: Schematic model integrating clinicopathological correlations and the pro-metastatic role of CAV1 in gallbladder cancer.
Analysis of tumor samples from gallbladder cancer (GBC) patients revealed that CAV1 expression increases, whereas E-cadherin (E-cadh) levels decrease within the pro-inflammatory microenvironment that characterizes this malignancy. A) Clinicopathological correlation shows that high CAV1 and low E-cadh levels are associated with a more aggressive GBC phenotype. B) Low CAV1 combined with preserved E-cadh expression reduces migration and invasion of GBC cells in vitro. C) Conversely, elevated CAV1 together with reduced E-cadh enhances in vitro migration and invasion. D) When these invasive GBC cells are injected into immunodeficient mice, CAV1 promotes metastatic dissemination, particularly under conditions of E-cadh loss. E) In vivo, these tumors exhibit increased metastatic burden and reduced survival, supporting a model in which CAV1 drives GBC progression in the context of diminished E-cadh expression.

7. Conclusions

According to our previous reported studies, CAV1 and E-cadh are present at the plasma membrane, where they form CAV1/E-cad complexes that sequester β-catenin. In doing so, they prevent translocation to the nucleus and β-catenin/Tcf-Lef-dependent transcription of genes, including survivin and COX-2. Alternatively, in the absence of E-cadh, CAV1 promotes migration, invasion and metastasis. Consistent with the results from those studies evaluating effects of CAV1 in different cancer cell lines, here elevated CAV1 levels were correlated with unfavourable clinicopathological features and poor survival in gall bladder cancer patients, as well as enhanced migration, invasion, and metastasis of gallbladder cancer cells, suggesting for first time important roles for CAV1 in this specific pathology and as a potential therapeutic target for the treatment of aggressive gallbladder cancer.

8. Acknowledgements

FONDAP Project 15130011 and FONDAP Continuation #1523A0008 (AFGQ); FONDECYT projects 1210644 and 1251598 (AFGQ), 1211223 (LLG); ANID PAI 77190041(LO); and ANID PhD student fellowships (LO).

References

1. Stinton LM, Shaffer EA. Epidemiology of Gallbladder Disease: Cholelithiasis and Cancer. Gut Liver. 2012; 6: 172-187.
2. Villanueva Olivares L. Cancer of the gallbladder-Chilean statistics. Ecancermedicalscience. 2016; 10.
3. Bertran E, Heise K, Andia ME, Ferreccio C. Gallbladder cancer: Incidence and survival in a high-risk area of Chile. Int J Cancer. 2010; 127: 2446-2454.
4. Koshiol J, Van De Wyngard V, McGee EE, Cook P, Pfeiffer RM. for the Chile BiLS Study Group. The Chile Biliary Longitudinal Study: A Gallstone Cohort. Am J Epidemiol. 2021; 190: 196-206.
5. Koca YS, Bulbul M, Barut I. The Diagnostic Roles of Cytokines in Hepatobiliary Cancers. BioMed Res Int. 2017; 2017: 1-5.
6. Cao H, Courchesne WE, Mastick CC. A Phosphotyrosine-dependent Protein Interaction Screen Reveals a Role for Phosphorylation of Caveolin-1 on Tyrosine 14. J Biol Chem. 2002; 277: 8771-8774.
7. Urra H, Torres VA, Ortiz RJ, Lobos L, Díaz MI. Caveolin-1-Enhanced Motility and Focal Adhesion Turnover Require Tyrosine-14 but Not Accumulation to the Rear in Metastatic Cancer Cells. PLoS ONE. 2012; 7: e33085.
8. Nunez-Wehinger S, Ortiz RJ, Diaz N, Diaz J, Lobos-Gonzalez L, Quest AFG. Caveolin-1 in Cell Migration and Metastasis. Curr Mol Med. 2014; 14: 255-274.
9. Quest A, Lobos-Gonzalez L, Nunez S, Sanhueza C, Fernandez J-G, Aguirre A, Rodriguez D, Leyton L, Torres V. The Caveolin-1 Connection to Cell Death and Survival. Curr Mol Med 2013; 13: 266-281.
10. Fernández MA, Albor C, Ingelmo-Torres M, Nixon SJ, Ferguson C. Caveolin-1 Is Essential for Liver Regeneration. Science. 2006; 313: 1628-1632.
11. Ortiz R, Díaz J, Díaz-Valdivia N, Martínez S, Simón L. Src-family kinase inhibitors block early steps of caveolin-1-enhanced lung metastasis by melanoma cells. Biochem Pharmacol. 2020; 177: 113941.
12. Bender F, Montoya M, Monardes V, Leyton L, Quest AFG. Caveolae and caveolae-like membrane domains in cellular signaling and disease: Identification of downstream targets for the tumor suppressor protein caveolin-1. Biol Res. 2002; 35.
13. Torres VA, Tapia JC, Rodríguez DA, Párraga M. Caveolin-1 controls cell proliferation and cell death by suppressing expression of the inhibitor of apoptosis protein survivin. J Cell Sci. 2006; 119: 1812-1823.
14. Torres VA, Tapia JC, Rodriguez DA, Lladser A, Arredondo C, Leyton L, Quest AFG. E-Cadherin Is Required for Caveolin-1-Mediated Down-Regulation of the Inhibitor of Apoptosis Protein Survivin via Reduced β-Catenin-Tcf/Lef-Dependent Transcription. Mol Cell Biol. 2007; 27: 7703-7717.
15. Rodriguez DA, Tapia JC, Fernandez JG, Torres VA, Muñoz N. Caveolin-1–mediated Suppression of Cyclooxygenase-2 via a β-catenin-Tcf/Lef–dependent Transcriptional Mechanism Reduced Prostaglandin E2 Production and Survivin Expression. Mol Biol Cell. 2009; 20: 2297-2310.
16. Fernández JG, Rodríguez DA, Valenzuela M, Calderon C. Survivin expression promotes VEGF-induced tumour angiogenesis via PI3K/Akt enhanced β-catenin/Tcf-Lef dependent transcription. Mol Cancer. 2014; 13: 209.
17. Sun G, Wu J, Wu J, Pan Y, Jin R. Caveolin-1, E-cadherin and β-catenin in gastric carcinoma, precancerous tissues and chronic non-atrophic gastritis. Chin J Cancer Res. 2012; 24: 23-28.
18. Felicetti F, Parolini I, Bottero L, Fecchi K, Errico MC, Raggi C, Biffoni M. Caveolin-1 tumor-promoting role in human melanoma. Int J Cancer. 2009; 125: 1514-1522.
19. Lobos-Gonzalez L, Aguilar-Guzmán L, Fernandez JG, Muñoz N. Caveolin-1 is a risk factor for postsurgery metastasis in preclinical melanoma models. Melanoma Res. 2014; 24: 108-119.
20. Díaz-Valdivia NI, Calderón CC, Díaz JE, Lobos-González L, Sepulveda H. Anti-neoplastic drugs increase caveolin-1-dependent migration, invasion and metastasis of cancer cells. Oncotarget. 2017; 8: 111943–111965.
21. Díaz-Valdivia NI, Díaz J, Contreras P, Campos A, Rojas-Celis V. The non-receptor tyrosine phosphatase type 14 blocks caveolin-1-enhanced cancer cell metastasis. Oncogene. 2020; 39: 3693-3709.
22. Li S, Seitz R, Lisanti MP. Phosphorylation of Caveolin by Src Tyrosine Kinases. J Biol Chem. 1996; 271: 3863-3868.
23. Goetz JG, Joshi B, Lajoie P, Strugnell SS, Scudamore T, Kojic LD, Nabi IR. Concerted regulation of focal adhesion dynamics by galectin-3 and tyrosine-phosphorylated caveolin-1. J Cell Biol. 2008; 180: 1261-1275.
24. Silva P, Soto N, Díaz J, Mendoza P, Díaz N, Quest AFG, Torres VA. Down-regulation of Rab5 decreases characteristics associated with maintenance of cell transformation. Biochem Biophys Res Commun. 2015; 464: 642-646.
25. Mendoza P, Díaz J, Silva P, Torres VA. Rab5 activation as a tumor cell migration switch. Small GTPases. 2014; 5.
26. Hjaltadóttir K, Haraldsdóttir KH, Möller PH. Gallstones – review. Læknablaðið. 2020; 106: 464-472.
27. Lobos-González L, Bustos R, Campos A, Silva V, Silva V, Jeldes E, Salomon C. Exosomes released upon mitochondrial ASncmtRNA

knockdown reduce tumorigenic properties of malignant breast cancer cells. Sci Rep. 2020; 10: 343.

28. Zhang L, Ma L, Li J, Lei J, Chen J, Yu C. VE-cadherin N -glycosylation modified by N -acetylglucosaminyltransferase V regulates VE-cadherin–β-catenin interaction and monocyte adhesion. Exp Physiol. 2021; 106: 1869-1877.
29. Lobos-González L, Aguilar L, Diaz J, Diaz N, Urra H. E-cadherin determines C aveolin-1 tumor suppression or metastasis enhancing function in melanoma cells. Pigment Cell Melanoma Res. 2013; 26: 555-570.
30. Espinoza JA, García P, Bizama C, Leal JL, Riquelme I, Weber H. Low expression of equilibrative nucleoside transporter 1 is associated with poor prognosis in chemotherapy-naïve pT2 gallbladder adenocarcinoma patients. Histopathology. 2016; 68: 722–728.
31. Bender FC, Reymond MA, Bron C, Quest AF. Caveolin-1 levels are down-regulated in human colon tumors, and ectopic expression of caveolin-1 in colon carcinoma cell lines reduces cell tumorigenicity. Cancer Res. 2000; 60: 5870-5878.
32. Stewart SA, Dykxhoorn DM, Palliser D, Mizuno H, Yu EY. Lentivirus-delivered stable gene silencing by RNAi in primary cells. RNA N Y N. 2003; 9: 493-501.
33. Acuña RA, Varas-Godoy M, Herrera-Sepulveda D, Retamal MA. Connexin46 Expression Enhances Cancer Stem Cell and Epithelial-to-Mesenchymal Transition Characteristics of Human Breast Cancer MCF-7 Cells. Int J Mol Sci. 2021; 22: 12604.
34. R Core Team. R: A Language and Environment for Statistical Computing. 2022.
35. Seshan V, Whiting K. clinfun: Clinical Trial Design and Data Analysis Functions. 2023.
36. Díaz J, Mendoza P, Ortiz R, Díaz N, Leyton L, Stupack D. Rab5 is required for Caveolin-1-enhanced Rac1 activation, migration and invasion of metastatic cancer cells. J Cell Sci 2014; 2401–2406.
37. Mendoza P, Ortiz R, Díaz J, Quest AFG, Leyton L, Stupack D, Torres VA. Rab5 Activation Promotes Focal Adhesion Disassembly, Migration and Invasiveness of Tumor Cells. J Cell Sci. 2013; 3835-3847.
38. Lobos-González L, Silva V, Araya M, Restovic F, Echenique J. Targeting antisense mitochondrial ncRNAs inhibits murine melanoma tumor growth and metastasis through reduction in survival and invasion factors. Oncotarget. 2016; 7: 58331-58350.
39. Ortiz R, Díaz J, Díaz N, Lobos-Gonzalez L, Cárdenas A. Extracellular matrix-specific Caveolin-1 phosphorylation on tyrosine 14 is linked to augmented melanoma metastasis but not tumorigenesis. Oncotarget. 2016; 7: 40571–40593.
40. Huilcaman R, Campos A, Contreras P, Simón L, Varas-Godoy M. Inclusion of AVβ3 integrin into extracellular vesicles in a caveolin-1 tyrosine-14- phosphorylation dependent manner and subsequent transfer to recipient melanoma cells promotes migration, invasion and metastasis. Cell Commun Signal. 2025; 23: 139.
41. Yang Q-E. Human cancer xenografts in immunocompromised mice provide an advanced genuine tumor model for research and drug development-A revisit of murine models for human cancers. Biochim Biophys Acta Gen Subj. 2021; 1865: 129929.
42. Gama A, Seixas F, Pires M dos A, Schmitt F, Payan-Carreira R. Disruption of E-Cadherin Pattern in Uterine and Mammary Tumours. In: McWilliam J, editor. Cadherins: Types, Structure and Functions. New York: Nova Science Publishers, Inc. 2020; 129-161.